DEVELOPMENT OF SCAR MARKER LINKED TO A ROOT-KNOT NEMATODE RESISTANT GENE IN PEANUT

A Thesis

by

HEE JEONG YANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Plant Pathology

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ABSTRACT

Development of SCAR Marker Linked to a Root-Knot

Nematode Resistant Gene in Peanut. (August 2004)

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Root-knot disease caused by *Meloidogyne spp.* is the most important nematode disease of peanut. Even though many management strategies have been applied to control this disease on peanut, resistance is the most recommendable. Marker-assisted selection has been used as a useful tool for screening of resistant individuals in segregating populations. However, it requires many laborious steps. Thus, there is a need for PCR - based markers, which are more practical, rapid, and efficient.

In this study, we tried to develop a SCAR marker linked to root-knot nematode resistance locus in peanut based on the RFLP marker R2430E. The entire sequence of R2430E was 2217 bp and contained one putative open reading frame (ORF) of 713 nucleotides. Thirteen primers including 5 forward and 8 reverse primers were synthesized to sequence the entireR2430E. Based on the results of BLAST searches, R2430E appeared to encode an AAA ATPase containing von Willebrand factor type A (VWA) domain from *Magnetococcus* sp. MC-1 (106 bits).

To determine if there is a portion of the R2430E that hybridizes only to a band co-segregating with the resistance locus, we generated 4 probes spanning different parts of the gene. Southern analysis using these probes revealed identical banding patterns for each probe. Therefore, we concluded that there is very limited if any sequence polymorphism between different alleles detected by the R2430E probe. Additionally, this conclusion is supported by the experiment in which we tested 25 primer pairs derived from the R2430E using genomic DNA from both resistance and susceptible genotypes. In this experiment, all primer pairs amplified identical PCR fragments, suggesting again that there is little or no sequence divergence between putative alleles as differentiated by southern blotting.

To identify possible single nucleotide polymorphisms (SNPs) between polymorphic R2430E RFLP bands, we cloned several fragments that span the entire R2430E transcribed sequence. Surprisingly, no SNPs were identified in the transcribed region of this gene. We propose that polymorphism detected by this RFLP marker is outside of the R2430E.

To my parents,

husband, Jae-Min, and my son, Myung-Hyun,

who have been my biggest supporters to complete this thesis

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Peanut (*Arachis hypogaea* L.) is cultivated mostly in tropical and sub-tropical climates. India and China are the largest peanut producers in the world; 13.5 million ha are grown in Asia, 5.3 million ha in Africa, 1.2 million ha in the Americas, and 0.1 million ha in other parts of the world (Carley and Fletcher, 1995). Texas is the second peanut producing state in the United States. In 2001, peanut was harvested from a total of 219,680 ha and yielded 2,770 kg/ha in Texas. Total peanut production was 608,372,000 kg (R. Lemon, College Station, TX, pers. comm.).

Peanut is an economically important crop that is grown as a major oilseed and also for human consumption. Additionally, the foliage is an important fodder crop and the meal remaining after oil extraction is an important source of animal feed. Peanut is susceptible to numerous foliar and soil-borne pathogens. The most global peanut diseases are early and late leaf spots and rust caused by *Cercospora arachidicola* Hori, *Cercosporidium personatum* Deighton, and *Puccinia arachidis* Speg. respectively (Stalker, 1997). In addition, *Sclerotium rolfsii*, which causes stem rot, *Sclerotinia minor* Jagger, which causes sclerotinia blight, several *Pythium* spp., and *Rhizoctonia solani* are soil-borne pathogens that cause important peanut diseases (Morris Porter et al., 1984).

This thesis follows the style and format of the Journal of Nematology.

Root-knot nematodes (*Meloidogyne* spp.) are the most important nematode pathogens of peanut (*Arachis hypogaea* L.).

Root-knot nematodes, *Meloidogyne* spp., are the most important biotropic plant - parasitic nematodes in agriculture. They are distributed worldwide and cause severe loss of crop yield (Sasser and Freckman, 1987). Estimated annual peanut yield losses caused by *M. arenaria* are 8 %, 5.5 %, and 3 % for Alabama, Georgia, and Texas, respectively (Koenning et al., 1999; Minton and Baujard, 1990). The three major species attacking peanut are *M. arenaria, M. hapla,* and *M. javanica. Meloidogyne arenaria* is the most prevalent species in Alabama, Florida, Georgia, and Texas (Ingram, 1980; Motsinger et al., 1976; Wheeler and Starr, 1987).

The life cycle of the root-knot nematode consists of the egg, four juvenile stages, and the adult. Second-stage juveniles (J2) hatch from the egg. These infective J2s migrate in soil and invade plant root-tips. J2s in the plant root migrate intercellularly through the cortical tissue and establish a feeding site composed of three to five altered host cells, called giant cells, in the vascular tissue. Giant cells are multinucleated, have dense cytoplasm, elevated rates of metabolism, and altered cell wall structure. These cells are the permanent feeding site for the nematode. The J2 become sedentary, molt to the J3 stage after feeding on a susceptible host, and enlarge as they go through the J4 stage, and then become adults. These mature adults lay eggs on the gall surface. Eggs are able to survive in unfavorable conditions in the soil for many months. It takes 20 to 60 days to complete the life cycle of root-knot nematode (Hussey et al., 2001; Niebel et al., 1994). The damage caused by root-knot nematodes leads to serious economic loss. Symptoms

of nematode infection are retarded growth; galling of root, pegs and pods; and wilting (Jung et al., 1998).

Many management tactics are available for management of root-knot nematodes on peanut, including crop rotation, biological control, planting nematode-resistant varieties, and nematicide application. However, each management tactic has limitations and none are sufficient to provide complete control. Crop rotation can be effective in decreasing the potential for substantial yield losses (Luc et al., 1990; Whitehead, 1998) and reducing nematode populations for the short-term. Cotton has been reported to be an effective rotation crop with peanut to reduce nematode soil populations (Rodriguez-Kabana et al., 1991). In addition, grasses such as bahiagrass, bermudagrass, millet, and sorghum have been used as rotation crops with peanut (Kinloch, 2001). Several biological control agents effective against root-knot nematode have been reported. *Pasteuria* spp. is one of the most effective biological agents that parasitizes *Meloidogyne* spp. (Dickson et al., 1991; Sayre and Starr, 1985). Nematodes parasitized by *Pasteria* spp. were still alive and develop to the reduced adult stage, but the number of eggs produced by infected females is greatly or completely reduced (Mankau, 1980; Sayre, 1980). Unfortunately, effective use and application of biological agents such as *Pasteria* spp. have not been developed. Nematicides can be used with some effectiveness but at a high management cost. Public concerns for nematicide residues in the environment have resulted in increased restrictions on their use. Thus, use of resistant cultivars has become a more attractive and more desirable alternative (Zijlstra et al., 2000). There are many advantages to resistant cultivars as a tactic for management of root-knot nematodes.

They do not require advanced technology, leave no toxic residues in environment, and are usually cost effective. Resistance is considered by some to be the most effective management to protect peanut from economic loss due to parasitism by root-knot nematodes (Trudgill, 1991).

Resistance to nematodes is defined as the ability of the plant to reduce and overcome nematode development and reproduction (Cook and Evans, 1987). Some resistance genes induce a local hypersensitive response (HR), which results in rapid, localized necrosis to prevent pathogen development; increase in peroxidase activity; and lignin deposition (Jung et al., 1998). In many cases, nematode - resistance genes have been identified by mapping and cloning using molecular markers, which are tightly linked to the resistance gene. Examples of resistance genes that have been well studied are *Gro1*, *H1, Gpa2* for resistance to the cyst nematode (*Globodera* spp.) in potato (Ballvora et al., 1995; Leister et al., 1996; Niewohner et al., 1995); *rhg1* and *Rhg4* from soybean for resistance to *Heterodera glycines* (Concibidio et al., 1997; Webb et al., 1995); *Mi-1* and *Mi-3* from tomato for resistance to *M. incognita, M. arenaria,* and *M. javanica.* Two genes in peanut, *Mae* and *Mag,* from *A. cardenasii* reduced nematode egg number and restrict gall formation (Garcia et al., 1996). All these resistance genes have been mapped with molecular markers such as RFLP, AFLP, and RAPD.

Currently, two peanut cultivars resistant to root-knot nematode are available. In 1999, the first peanut cultivar ('COAN') with resistance to the *M. arenaria* and *M. javanica* was released by the Texas Agricultural Experiment Station (Simpson and Starr, 2001). The resistance in COAN originated from wild *Arachis cardenasii* by introgression into

A. hypogaea (Simpson, 1991). The germplasm line TxAG-6 was generated by interspecific hybridization [*A. batizocoi* x (*A. cardenasii* x *A. diogoi*)]^{4X} (Simpson, 1991). The resistance was then introgressed into *A. hypogaea* by a backcross program with a component line UF 439-16-10-3-2 of cv. Florunner as the recurrent parent. Resistance of COAN to *M. arenaria* is expressed as a reduction in nematode reproduction. Although nematodes invade the roots of COAN, most emigrate from the roots and the few that remain in the roots develop to reproductive adults (Bendezu and Starr, 2003). No hypersensitive reaction has been observed in these resistant genotypes. The second peanut cultivar resistant to *M. arenaria*, NemaTAM, was released in 2002. (Simpson et al., 2003). NemaTAM was derived from the same backcross introgression pathway as COAN; COAN was selected from the fifth backcross generation whereas NemaTAM was selected from the seventh backcross generation. The resistance in COAN and NemaTAM is controlled by a single dominant gene (Burow et al., 1996; Choi et al., 1999; Church et al., 2000) and restriction fragment length polymorphism (RFLP) markers linked to the gene have been identified (Burow et al., 2001; Choi et al., 1999).

Molecular markers such as RFLPs, amplified-fragment length polymorphisms (AFLP), random-amplified polymorphic DNA (RAPD), sequence-characterized amplified region (SCAR), and sequence-tagged sites (STS) can facilitate breeding programs via marker-assisted selection. DNA markers are most useful as tools in the transfer of single genes from a donor to a recipient line (Ribaut and Hoisington, 1998).

Classical breeding techniques have contributed to the development of resistant varieties for a long time, but these techniques are laborious, expensive, and take a long time to develop a new resistant cultivar. Using DNA marker technologies in breeding programs can improve these weak points of classical backcross programs (Hussey et al., 2001). DNA marker technologies for marker-assisted selection can be economic, reliable, and efficient methods for selection of resistant individuals in segregating populations. The recurrent genome can be recovered faster than by classical backcross programs using phenotypic selection procedures. Moreover, marker technologies are time effective since we do not need to grow the plants through their life cycle and await phenotypic evaluations.

Many DNA markers linked to the root-knot nematode resistance gene have been reported in wild peanut species. RAPD marker (Z3/265) linked to resistance genes *Mag* and *Mae* to root-knot nematode *M. arenaria* in peanut has been identified and was converted into a SCAR marker (Garcia et al., 1996). Burow et al. (1996) reported three RAPD markers, *RKN410*, *RKN440*, and *RKN229* linked to the root-knot nematode resistance gene derived from *A. cardenasii.* A genetic map of cultivated peanut (*Arachis hypogaea* L.) was achieved by RFLP analysis (Burow et al., 2001). A synthetic amphidiploid, TxAG-6 (Simpson 1991), which was developed through the cross [*A. batizocoi* x (*A. cardenasii* x *A. diogoi*)]^{4X} was used as a donor to introduce the root-knot nematode resistance gene into cultivated peanut. Three hundred seventy RFLP loci were mapped (Burow et al., 2001). Based on this study, Choi et al. (1999) identified two useful RFLP markers, R2430E and R2545E. RFLP loci R2430E and R2545E showed

quite distinct bands on resistant and susceptible alleles and were easy to score for genetic condition, homozygosity vs. heterozygosity, in individual plants. RFLP locus R2430E was 4.2 centiMorgans (cM) from the resistance locus (Choi et al., 1999). RFLP locus R2430E was derived from *A. cardenasii* and maps to linkage group 1 (Burow et al., 2001). Church et al. (2000) reported the efficiency of marker-assisted selection using two RFLP markers, R2430E and R2545 to identify individuals homozygous for resistance to *M. arenaria* in segregating populations of peanut.

Although molecular markers are utilized to select resistance in individual plants, RFLP or RAPD analysis is impractical for routine identification directly from plants. RFLP analysis requires several time-consuming steps, including DNA extraction from plant tissue, digestion, electrophoresis, and southern hybridization. RAPD analysis often lacks reproducibility and thus is not sufficiently robust for routine marker-assisted selection programs. Therefore, there is a need to develop a reliable, robust PCR-based marker upon which a more efficient, reliable, relatively simple marker-assisted selection procedure can be based (Paran et al., 1993). Various types of PCR-based markers have been developed; amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR, also called microsatellites or short tandem repeats, STR), and sequence characterized amplified regions (SCARs) (Harry et al., 1998). Usually, PCR-based markers can be developed by designing either specific primers based on known DNA sequence or with arbitrary primers. The former are allele specific primers and the other is used for development of random amplified polymorphism (RAPD) (Williams et al., 1990). RAPDs are able to quickly and simply monitor marker loci but this technique is

not reliable because RAPD is very sensitive to the reaction conditions. Sequence-characterized amplified regions (SCARs) can be a solution for the problem of irreproducibility (Paran et al., 1993). SCAR represent a single defined genomic DNA locus and may be identified by PCR amplification using sequence specific primer pairs. SCAR primers can be synthesized based on single nucleotide polymorphisms (SNPs) and can be very reliable and powerful DNA markers for selecting resistant individuals. SNPs are the most prevalent form of genetic variation and occur by changing a single nucleotide in a genome sequence. SNPs may serve as useful markers by themselves as well as a powerful tool for genome mapping (Batley et al., 2003). Research on SNPs has gained an importance in human disease since many SNPs have been found in the human genome. Most SNPs involve the replacement of cytosine (C) with thymine (T). In plants, SNPs are useful as genetic markers and can be used in breeding programs. According to previous research, SNPs occur more abundantly in plants than in the human genome (Germano and Klein, 1999; Coryell et al., 1999). SCARs also can be used as a physical landmark in the genome (Paran et al., 1993).

In many cases, SCAR markers have been developed by converting dominant RAPD markers. SCARs can be developed by cloning and sequencing of polymorphic RAPD fragment, and then SCAR primers can be designed based on the insert sequence containing the RAPD primer. Conversion from RAPDs to SCARs allows enhancing of reliability and efficiency. Garcia et al. (1996) developed the SCAR marker (SCZ3) linked to nematode resistance gene in *Arachis hypogaea* by conversion of RAPD marker (Z3/265). Unfortunately, the linkage between SCZ3 and the resistance locus is not

sufficient for a robust marker-assisted selection procedure. RAPD marker $OPG17_{450}$ linked to the *Ns* gene in potato was transformed into SCAR markers $SCG17_{321}$ and $SCG17_{448}$ (Marczewsk et al., 2001) and SCAR markers linked to the *Pm21* gene conferring resistance to powdery mildew in common wheat were developed by the same method (Liu et al., 1999). SCARs can also be developed from RFLP probes (Paran et al., 1993).

The goal of this study was to develop SCAR markers linked to the gene for resistance to *M. arenaria* in peanut using RFLP locus R2430E (Choi et al., 1999).

CHAPTER II

MATERIALS AND METHODS

Plant materials

All studies used the near-isogenic susceptible and resistant peanut genotypes, Florunner component line, UF439-16-10-3-2 and NemaTAM, respectively. Peanut seeds were planted in a peat - based medium in 38 cm × 53 cm flats of the Texas A&M University greenhouse (Agronomy Rd,). Irrigation was applied as needed.

Plant genomic DNA extraction

Peanut genomic DNA was extracted from resistant and susceptible genotypes by the CTAB extraction method (Chen et al., 1999). Unexpanded tetrafoliolate leaves were collected from 1 to 2 - week-old plants. The leaves were placed individually in 1.5-ml Eppendorf tubes and stored at -80 ^{o}C. The frozen leaf tissue was ground initially with a small spatula, and then ground with a plastic pestle mounted in an electric drill. Immediately after grinding, 700 µl of warm (65 ^{o}C) extraction buffer [2 % w/v CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris- HCl, pH 8.0, 2% w/v polynvinylpyrrolidone (PVP-40), 5.0 mM ascorbic acid, 4.0 mM diethyldithiocarbamic acid (Doyle and Doyle, 1990)] were added to the tissue powder. Three µl of 2-mercaptoehanol and 7 µl of RNase A (20 mg/ml) were added to each sample and the mixture was inverted gently and incubated at 65 ^{o}C for 5 minutes. A chloroform: isoamyl alcohol mixture (24: 1 v/v)

was added to each tube, and the mixture was shaken by hand and then centrifuged at 14,000 ×g for 10 minutes at room temperature. The upper, DNA-containing phase was transferred to a new Eppendorf tube. DNA was precipitated by adding 0.7 volumes of cold isopropanol, mixed, and immediately centrifuged at 14,000 ×g for 5 minutes. The DNA pellet was washed three times with 70 % ethanol. After the last wash, the DNA pellet was centrifuged at 14,000 ×g for 5 minutes and the supernatant was discarded. The resulting DNA pellet was air-dried and the DNA was dissolved in 50 µl of TE.

Sequencing of RFLP locus R2430E

cDNA clone R2430E was obtained from A. H. Paterson (University of Georgia) as an insert in pBluescript II SK^- maintained in *E. coli*. The cells were cultured in Luria – Bertani medium (LB) containing ampicillin (75µl/ml) overnight at 37 ^oC. Plasmid DNA to be used as a template for sequencing was extracted from the cultures using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA). The concentration of the plasmid DNA was measured using Nanodrop (Gene Technology Lab Technologies Inc.). The entire cDNA clone R2430E was sequenced using the ABI prism Bigdye terminator sequencing kit following the manufacturer's instruction (Applied Biosystems, Foster City, CA). T3 primer (5'-AATTAACCCTCACTAAAGGG-3') and T7 primer (5'-TAATACGACTCACTATAGGG -3') were used to obtain initial sequences in both directions and new primers were synthesized based on the sequencing data and used to complete the entire R2430E sequence. All five forward primers and eight reverse primers were synthesized by Primer 3 program (http://www-genome.wi.mit.edu/cgi-

bin/primer/primer3_www.cgi) (Table 1). All primers were 19 to 22 mers length with 36 to 60 % of GC content, and a Tm of 56 to 65 ^{o}C. Automated sequencing was performed by the Gene Technology Lab (GTL, Texas A&M University) and analyzed using the Sequencher program (Gene Codes Corporation, Michigan). The sequencher program was used under condition of 85 % of minimum match and 20 % of minimum overlap to assemble all the sequences. Sequences, which were derived from different primer pairs were overlapped at least both strands or multiple fragments at the same direction to make a contig.

The R2430E sequence was subjected to BLAST searches within the GenBank database (http://www.ncbi.nle.nig.gov/) at the National Center for Biotechnology Information (NCBI) to determine basic similarity with other sequences in the data base.

PCR amplification

PCR reactions were carried out in 25 µl volumes that contained 12.5 µl of REDTag ReadyMix PCR Reaction Mix (Sigma, St. Louis, MO), 1 µl of genomic DNA (100 ng/µl), 1.25 µl of each primer (10 µM), and 9 µl of sterile water. Amplification was carried out under the following conditions: 94 ^{o}C for 4 minutes; 35 cycles at 94 ^{o}C for 45 seconds; 50 ^{o}C for 45 seconds; 72 ^{o}C for 1 to 3 minutes; and a final extension at 72 ^{o}C for 7 minutes. Extension time depended on the size of PCR products (1 minute of extension time / 1kb of expected PCR product). PCR products were electrophoresed in 1% agarose, stained with ethidium bromide (0.5 mg/ml), and the DNA bands visualized

Table 1. Primer sequences used for amplification and sequencing different portions of the cDNA probe R2430E.

Name	Sequence (5' → 3')	Position[1] (bp)	Size (mers)	*Tm* (^{o}C)	GC (%)
3H	TGATTCCCGAATTGGAAGAG	37 – 56	20	58.35	45
3L	TGCTCCTCTTCTTCAACCGT	648-667	20	60.4	50
RV1	AGTCTGCAGAAACGAGAAA	719 – 737	19	60.4	45
RV1-Brv	TCTGCTTTCTCAAGGCACTG	1149 – 1169	20	60.4	50
RV1-B	CAGTGCCTTGAGAAAGCAGA	1149 – 1169	20	60.4	50
3IB-aa	TCACATCTTGAGCCACTTCG	1385 – 1404	20	60.4	50
3IB-ab	ATCTTCAACCTTTTCCGCAA	1477 – 1496	20	56.3	40
3IB-a	TCCTTTACGGCAGCAGAGAT	1668 – 1687	20	60.4	50
3IB-b	CCAGTGTCTCTCTAACGGGC	1688 – 1707	20	64.5	60
5L	ATCTCTGCTGCCGTAAAGGA	1668 – 1687	20	58.35	50
IBRV2	AGCTAAACCAGTACAGAAGC	1804 – 1823	20	56.71	40
IBFW1	AACACTATATGCACCACAAC	2008 – 2027	20	56.3	40
5H	CACCCCTTTCCATTCAAACA	2061 – 2080	20	58.35	45
3 end	AAAGTCACAAAACCCATAGGAA	2175 – 2196	22	57.08	36.36

[1] Position in the R2430E sequence (5' → 3')

under UV light. PCR amplification was performed at least three times independently per primer set.

Plant subgenomic DNA preparation

Subgenomic DNA was obtained from genomic DNA. Genomic DNA from resistant and susceptible genotypes was extracted by the CTAB method (Chen et al., 1999) as described, and then 10 µg of genomic DNA was digested with EcoR I following the manufacturer's instruction (New England Biolabs, Beverly, MA). Digested DNA was

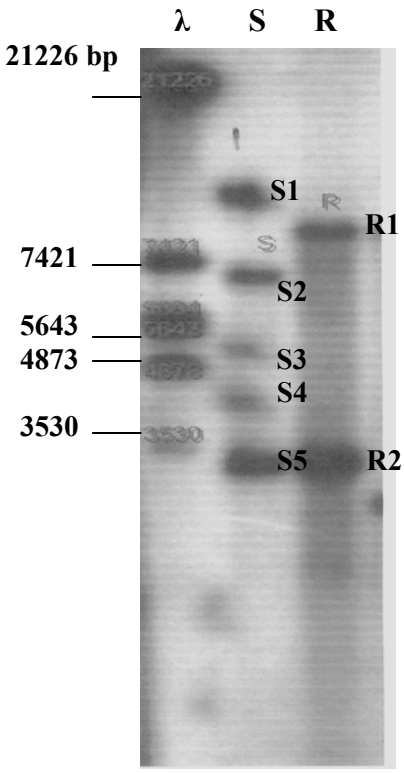

Fig. 1. The RFLP locus R2430E from Floruner and NemaTam. Lane 1: λ / EcoRI, Lane 2: Florunner (Susceptible), Lane 3: NemaTAM (Resistance).

separated electrophoretically on 0.8 % agarose gel (22 volts, overnight). Seven expected RFLP bands (Fig. 1) specific for resistant and susceptible genotypes probed with RFLP locus R2430E (unpubl. data) were excised from the gel and the DNA was extracted using the QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA).

Southern hybridization

Two µg of genomic DNA extracted by the CTAB method as described were digested with EcoR I according to the manufacturer's instructions (New England Biolabs, Beverly, MA) and then separated on a 0.8 % agarose gel (22 volts, overnight). The DNA was then southern blotted onto Hybond-N^+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's instructions and hybridized with radiolabeled probes. Southern hybridization was performed under high-stringency conditions (65 ^oC, multiple washes with 2 ×, 1 ×, 0.1 × SSC with 0.1% SDS) and routine procedures were carried out using standard protocols (Sambrook et al., 1989).

Analysis of single nucleotide polymorphisms

PCR was performed using peanut subgenomic DNA as a template. Five selected primer pairs were used to cover the entire R2430E sequence. Fifty ng of each subgenomic DNA (R1, R2, S1, S2 etc), which were derived from two resistant RFLP bands and five susceptible bands (Fig. 1) were used separately as templates. Other PCR conditions were as described previously. Three independent PCR reactions were performed to increase fidelity. All PCR products (7 templates × 5 primer pairs) were

cloned using the TOPO cloning kit (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions and every clone was sequenced in both directions using pUC/M13 reverse (5'-CAGGAAACAGCTATGAC-3'), forward primers (5'- GTTTTCCCAGTCACGAC-3') to identify single nucleotide polymorphisms (SNPs). Sequencing of the inserts was performed with three replications per each clone. Polymorphism of each clone was analyzed using the Sequencher program (Gene Codes Corporation, Michigan).

CHAPTER III

RESULTS

cDNA clone R2430E sequencing

The sequence of R2430E was determined by subsequent primer pairs, which were derived from the sequencing data and different portions of the sequence of R2430E were aligned into a contig for overlapping regions using Sequencher (Fig. 2). The complete sequence of cDNA clone R2430E was 2217 bp (Appendix) and contained one putative open reading frame, which was from 36 bp to 749 bp. This ORF encoded 237 amino acids.

The complete nucleotide sequence of R2430E and amino acid sequence of the putative ORF were submitted for homology searches (Table 2). A search for sequence homology against nucleotide databases showed low similarity to known genes. The *Arabidopsis thaliana* expressed protein (At3g05900) mRNA sequence showed the most significant alignment (E value $3e^{-}04$) to the nucleotide sequence of R2430E (56 bits). AAA ATPase containing von Willebrand factor type A domain showed the most significant alignment to the amino acid sequence of the putative ORF and nucleotide sequence translated in ORF (Table 2). The predicted protein sequence of the ORF homology showed high similarity to AAA ATPase containing von Willebrand factor type A (VWA) domain (106 bits, E-value 1e-21). Whereas, this protein showed low similarity to nucleotide sequence translated in ORF (41 bits, E-value 0.028).

Table 2. Putative alignments of nucleotide and protein sequence encoded by ORF of cDNA R2430E.

Algorithm	Significant alignments	Score (bits)	E value
BLASTN	Arabidopsis thaliana expressed protein (At3g05900)	56	3e-04
	Arabidopsis thaliana chromosome III BAC F10A16 genomic sequence	56	3e-04
BLASTP	COG5271: AAA ATPase containing von Willebrand factor type A domain [Magnetococcus sp. MC-1]	106	1e-21
	Poly E-rich protein [Helicobacter pylori 26695]	102	1e-20
	Neurofilament 3, medium; neurofilament, medium polypeptide [Mus musculus]	94	5e-18
	Antigen 332, putative [Plasmodium falciparum3D7]	94	7e-18
BLASTX	COG5271: AAA ATPase containing von Willebrand factor type A domain [Magnetococcus sp. MC-1]	41	0.028
	COG3025: Uncharacterized conserved protein [Methanosarcina barkeri]	41	0.028
	Vacuolar calcium binding protein [Raphanus sativus]	39	0.14
	Similar to zinc finger protein RIZ [Rattus norvegicus]	38	0.18

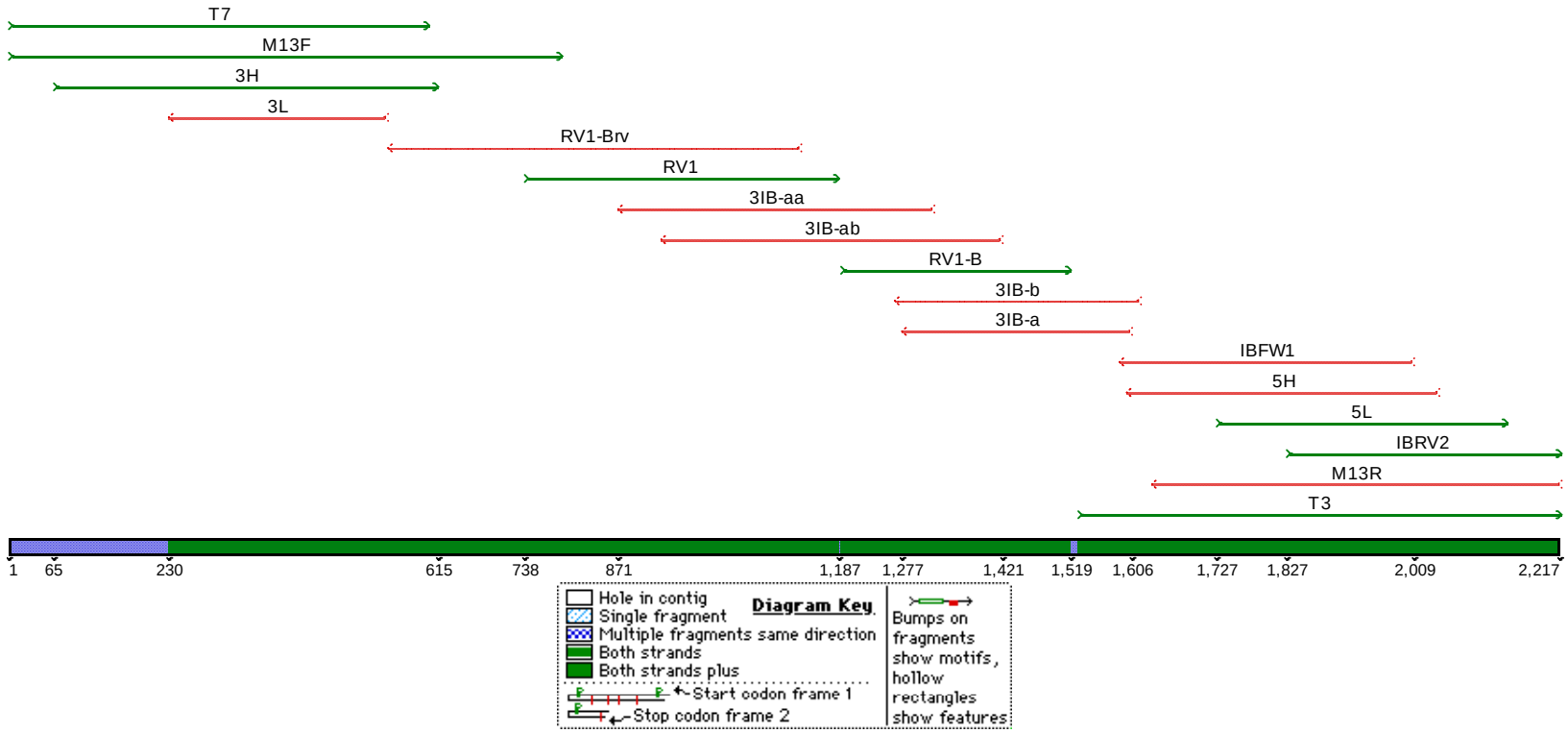

Fig. 2. Contig of R2430E. Green arrows indicate the sequences from the 5 forward primers designed, red arrows indicate the sequences from the 8 reverse primers designed in this study. Contig was generated by the Sequencher program. Sequences were overlapped at least both strands or multi fragments at same direction.

Southern analysis

Three additional probes, based on partial sequences of R2430E, were generated by PCR using different primer combinations and the cDNA clone R2430E as a template (Table 3, Fig 3). Probe A, which was amplified by primer pair 3H and 3L corresponded to the 5' end of R2430E. Probe B for 3' end was generated by primer pair 5L and 5H; probe C for middle region of R2430E was generated by primer pair RV1 and 3IB-a; and probe D for the entire R2430E was generated by primer pair T3 and T7. Amplified PCR fragments were purified using the QIAquick gel extraction Kit (QIAGEN Inc., Valencia, CA). Following southern analysis, we observed the same banding pattern with each of the different probes derived from R2430E (Fig. 4). There were two bands shown on both resistant and susceptible genotype respectively. The lower band (~ 3.5 kb) was common to both resistant and susceptible whereas, resistant individuals had a specific major band about 8 kb and the susceptible individuals had a major band about 7 kb. The observed RFLP corresponded to the previous reports by Choi et al. (1999) and Church et al. (2000). In this study, four probes, which were small fragments of R2430E were hybridized the same as the entire R2430E to the resistant locus. Thus, we could assume that the entire R2430E associates with the resistant locus.

Table 3. Probe characteristics for estimating which portion of R2430E hybridizes to RFLP bands from resistant and susceptible peanut genotypes.

	Forward primer	Reverse primer	Size (bp)	Position (bp)[1]
Probe A	3H	3L	630	37 – 667
Probe B	5L	5H	412	1668 - 2080
Probe C	RV1	3IB-a	968	719 - 1689
Probe D	T3	T7	2217	1 – 2217

[1] Position in the R2430E sequence (5'→ 3')

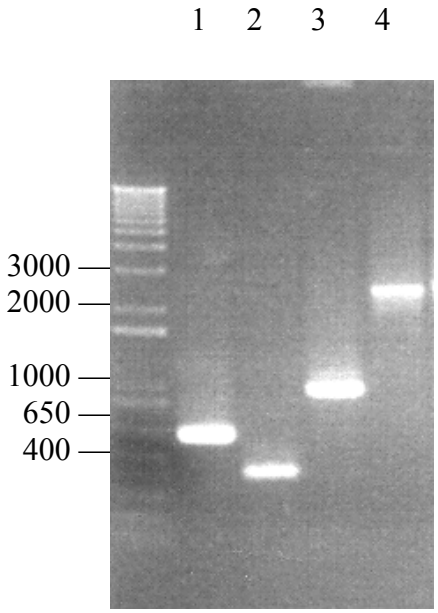

Fig. 3. Comparison of PCR amplification of segments of R2430E based on different primer pairs. Lane 1) probe A (630 bp) for 5'end of R2430E developed using primer pair 3H and 3L, Lane 2) probe B (412 bp) for 3'end of R2430E developed using primer pair 5L and 5H, Lane 3) probe C (968 bp) for middle part of R2430E developed using primer pair RV1 and 3IB-a, Lane 4) probe D (2217 bp) for entire R2430E developed using primer pair T3 and T7.

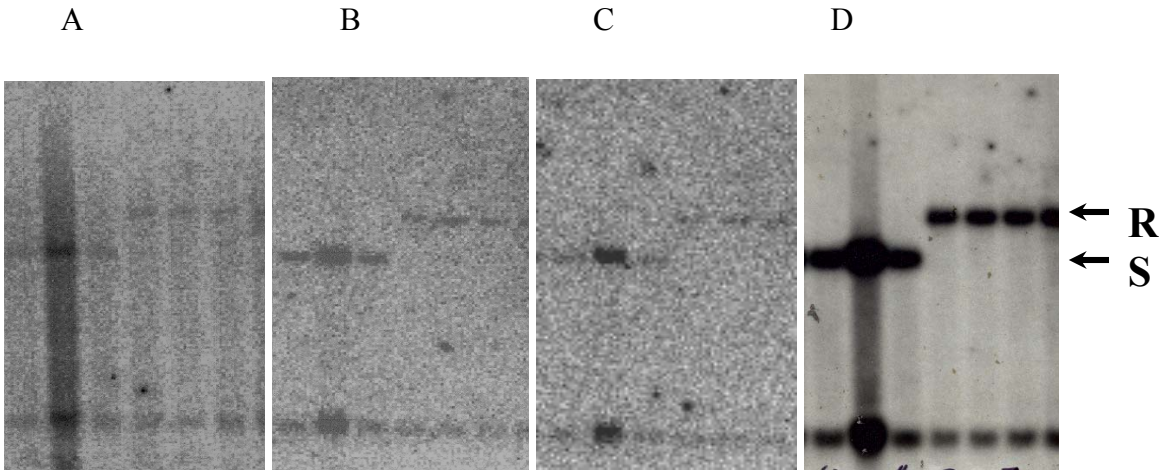

Fig. 4. Images of southern analysis of peanut genomic DNA extracted from NemaTAM and Florunner peanuts, digested with EcoR I, and probed with different portions of the cDNA R2430E. First Three lanes were Florunner (S), the other three lanes were NemaTAM (R). Upper band was for resistance, lower band was for susceptible and the bottom band was common for both resistance and susceptible. A) Probe A, which was the 5' end fragment of R2430E, B) probe B, which was the 3' end fragment of R2430E, C) probe C, which was the middle fragment of R2430E and D) probe D, which was the entire R2430E.

Test primer combinations with genomic DNA as a template

Twenty-five primer pairs, each derived from the sequence of R2430E, were used to amplify different portions of peanut genomic DNA from NemaTAM and Florunner as a template (Table 4). Each of the tested primer pairs generated a product of the size from template DNA from Florunner and NemaTAM peanut. No amplification product specific for the resistant or susceptible peanut cultivar was detected (Fig. 5).

Analysis of single nucleotide polymorphisms

Thirty-five PCR products were amplified using seven subgenomic DNAs, which were derived from the RFLP bands (Fig. 6) as a template and five primer pairs (7 templates × 5 primer pairs) (Table 5). Clone A was in the 5' end of R2430E and clone B, C, D, E were from the middle portion to 3' end of R2430E. All clones except clone A overlapped partially (Fig. 7).

Sequencing of all clones in both directions was carried out with three replication using three positive colonies per clone (35 clones × 3 positive colonies). Raw sequences for each clone were trimmed by hand and aligned into a contig. The entire sequence of R2430E was also assembled into the contig. Then, the sequences were analyzed for single nucleotide polymorphisms. When we compared the sequences, we focused on the sequence of the clone with the insert, which was derived from subgenomic DNA R1, unique specific RFLP band for resistance hybridized to R2430E.

Table 4. Sequences of primer pairs used to compare amplification products with template DNA from Florunner and NemaTAM peanut.

No	Forward Primer	Sequence (5' → 3')	Reverse Primer	Sequence (5' → 3')	Position (bp)	Size of amplified fragment (bp)
1	3H	TGATTCCCGAATTGGAAGAG	3L	TGCTCCTCTTCTTCAACCGT	37 - 667	630
2	3H	TGATTCCCGAATTGGAAGAG	RV1-Brv	TCTGCTTTCTCAAGGCACTG	37 – 1169	1132
3	3H	TGATTCCCGAATTGGAAGAG	3IB-aa	TCACATCTTGAGCCACTTCG	37 - 1404	1367
4	3H	TGATTCCCGAATTGGAAGAG	3IB-ab	ATCTTCAACCTTTTCCGCAA	37 – 1496	1459
5	3H	TGATTCCCGAATTGGAAGAG	3IB-a	TCCTTTACGGCAGCAGAGAT	37 – 1687	1650
6	3H	TGATTCCCGAATTGGAAGAG	3IB-b	CCAGTGTCTCTCTAACGGGC	37 – 1707	1670
7	3H	TGATTCCCGAATTGGAAGAG	IBFW1	AACACTATATGCACCACAAC	37 – 2027	1990
8	3H	TGATTCCCGAATTGGAAGAG	5H	CACCCCTTTCCATTCAAACA	37 – 2080	2043
9	RV1	AGTCTGCAGAAACCGAGGAA	RV1-Brv	TCTGCTTTCTCAAGGCACTG	719 – 1169	450
10	RV1	AGTCTGCAGAAACCGAGGAA	3IB-aa	TCACATCTTGAGCCACTTCG	719 – 1404	685
11	RV1	AGTCTGCAGAAACCGAGGAA	3IB-ab	ATCTTCAACCTTTTCCGCAA	719 – 1496	777
12	RV1	AGTCTGCAGAAACCGAGGAA	3IB-a	TCCTTTACGGCAGCAGAGAT	719 – 1687	968
13	RV1	AGTCTGCAGAAACCGAGGAA	3IB-b	CCAGTGTCTCTCTAACGGGC	719 – 1707	988
14	RV1	AGTCTGCAGAAACCGAGGAA	IBFW1	AACACTATATGCACCACAAC	719 – 2027	1308
15	RV1	AGTCTGCAGAAACCGAGGAA	5H	CACCCCTTTCCATTCAAACA	719 – 2080	1361
16	RV1-B	CAGTGCCTTGAGAAAGCAGA	3IB-aa	TCACATCTTGAGCCACTTCG	1159 – 1404	245
17	RV1-B	CAGTGCCTTGAGAAAGCAGA	3IB-ab	ATCTTCAACCTTTTCCGCAA	1159 – 1496	337
18	RV1-B	CAGTGCCTTGAGAAAGCAGA	3IB-a	TCCTTTACGGCAGCAGAGAT	1159 – 1687	528
19	RV1-B	CAGTGCCTTGAGAAAGCAGA	3IB-b	CCAGTGTCTCTCTAACGGGC	1159 – 1707	548
20	RV1-B	CAGTGCCTTGAGAAAGCAGA	IBFW1	AACACTATATGCACCACAAC	1159 – 2027	868
21	RV1-B	CAGTGCCTTGAGAAAGCAGA	5H	CACCCCTTTCCATTCAAACA	1159 – 2080	921
22	5L	ATCTCTGCTGCCGTAAAGGA	IBFW1	AACACTATATGCACCACAAC	1668 – 2027	359
23	5L	ATCTCTGCTGCCGTAAAGGA	5H	CACCCCTTTCCATTCAAACA	1668 – 2080	412
24	IBRV2	AGCTAAACCAGTTATAGAAGC	IBFW1	AACACTATATGCACCACAAC	1804 – 2027	223
25	IBRV2	AGCTAAACCAGTTATAGAAGC	5H	CACCCCTTTCCATTCAAACA	1804 - 2080	276

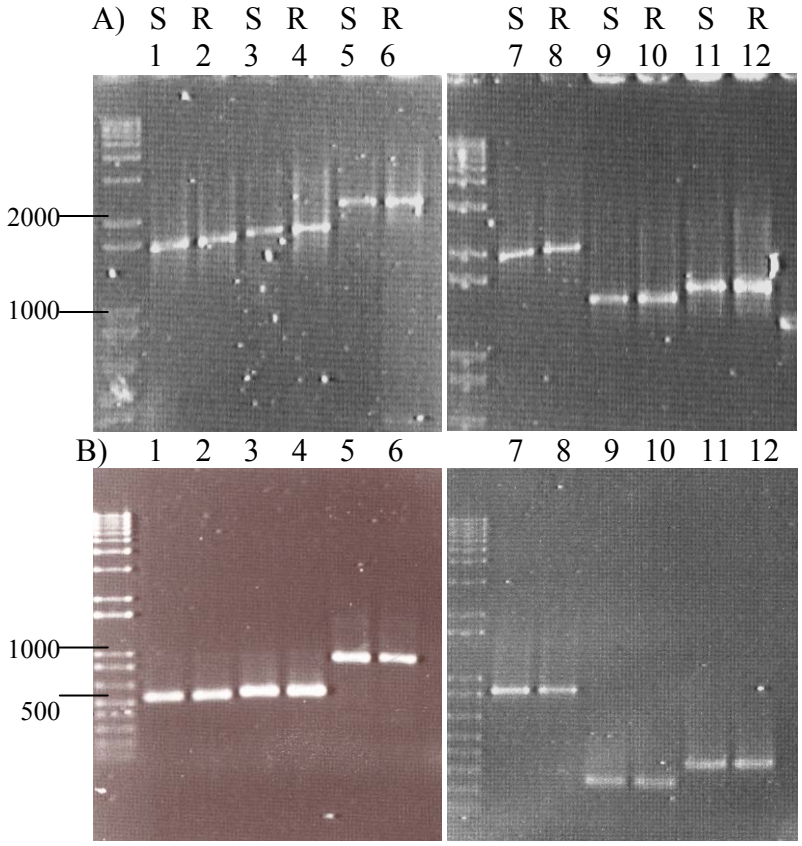

Fig. 5. Comparison of amplification products produced from different primer pairs and template DNA extracted from NemaTAM (R) and Florunner (S) peanut. A) Lanes 1 and 2 were from primer pair 3H / 3IBa, lanes 3 and 4 were from primer pair 3H / 3IBb, lanes 5 and 6 were from primer pair 3H / 5H, lanes 7 and 8 were from primer pair 3H / IBFW, lanes 9 and 10 were from primer pair 3H / 3IBaa, lanes 11 and 12 were from primer pair 3H / 3IBab. B) Lanes 1 and 2 were from primer pair RV1B / 3IBa, lanes 3 and 4 were from primer pair RV1B / 3IBb, lanes 5 and 6 were from primer pair RV1B / 5H, lanes 7 and 8 were from primer pair RV1B / IBFW, lanes 9 and 10 were from primer pair RV1B / 3IBaa, lanes 11 and 12 were from primer pair RV1B / 3IBab. See Table 4 for the sequence of each primer.

Several putative SNPs were found in each clone but most of them were not consistent except those in Clone E (Table 6). Four SNPs were found in Clone E and those were consistent through all the sequences of Clone E (Table 6). Each of the SNPs was due to nucleotide replacement or insertion/deletion. Guanine (G) in RFLP band R1 was replaced by adenine (A) on susceptible at 1924 bp (SNP 1). Cytosine (C) was replaced with thymine (T) at 1938 bp, SNP 2; guanine (G) was replaced with thymine (T) at 1978 bp, (SNP 3); and adenine (A) was inserted in the sequence of RFLP band R1 at 2110 bp, (SNP 4) (Fig. 8). To confirm whether those 4 SNPs found in clone E were true, we aligned the sequences of clone D and clone E together because these 2 clones were partially overlapped each other. However, SNP at the same position where 4 SNP found in clone E were not present in clone D (Fig. 9). Therefore, we concluded 4 SNP found in clone E were not true SNP and could not find any true SNP in the sequences of RFLP bands hybridized to R2430E.

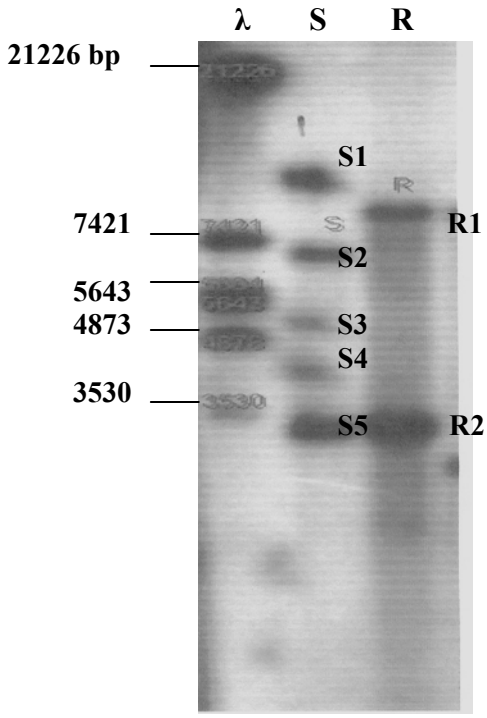

Fig. 6. The RFLP locus R2430E from Floruner and NemaTam. Lane 1: λ / EcoRI, Lane 2: Florunner (Susceptible), Lane 3: NemaTAM (Resistance).

Table 5. Clone characteristics.

Name	Forward primer	Reverse primer	Position(bp)	Size (bp)
Clone A	3H	3L	37 – 667	630
Clone B	RV1	3IB-ab	719 –1496	777
Clone C	RV1-B	3IB-b	1159 – 1707	548
Clone D	5L	IBFW1	1668 – 2027	359
Clone E	IBRV2	3 end	1804 – 2196	392

Table 6. Analysis of single nucleotide polymorphism (SNP) of clones in R2430E using RFLP bands.

Name	No. of colony well sequenced / Total No. of colonies tried to sequence	No. of putative SNPs found / Total No. of nt.	No. of consistent SNPs
Clone A	4/6 (67 %)	6/630	0
Clone B	21/21 (100 %)	34/777	0
Clone C	16/21 (76%)	7/548	0
Clone D	21/21 (100 %)	23/359	0
Clone E	17/21 (81 %)	12/392	4

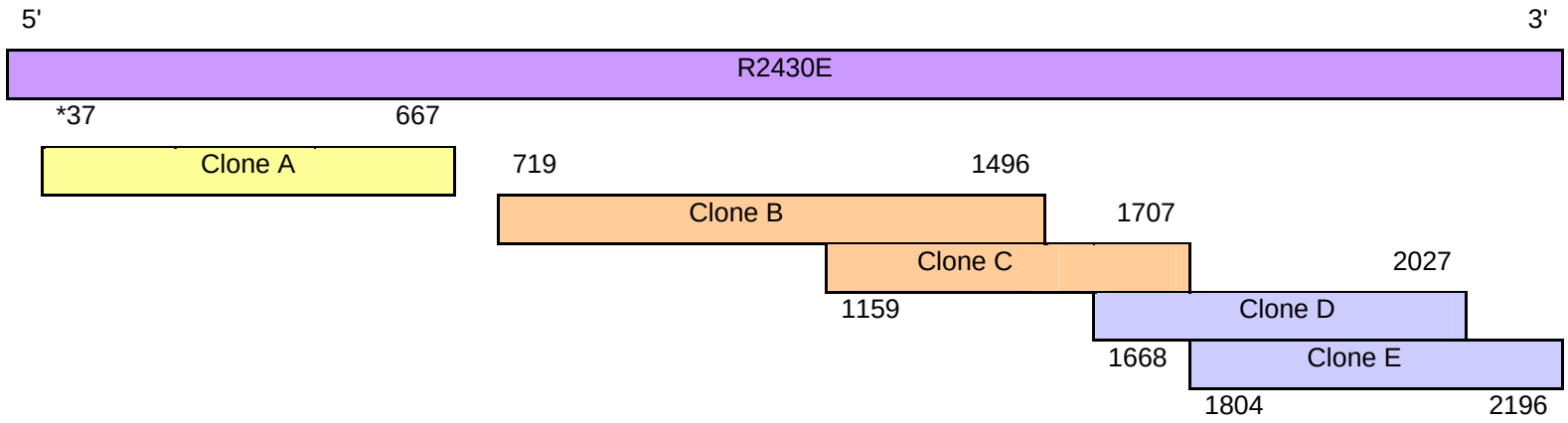

Fig. 7. The diagram of clones in R2430E. Each bar indicates each clone and position in R2430E. Clone A (630 bp) was in the 5' end of R2430E and developed by primer pair 3H and 3L. Clone B, C, D and E were from in the middle to the 3' end of R2430E. Clone B (777 bp) was developed by primer pair RV1 and 3IB-ab, clone C (548 bp) was developed by primer pair RV1-B and 3IB-b, clone D (359 bp) was developed by primer pair 5L and IBFW1, and clone E (392 bp) was developed by primer pair IBRV2 and 3 end. *: clone position (bp) in R2430E. See Table 4 for the location and sequence of each primer.

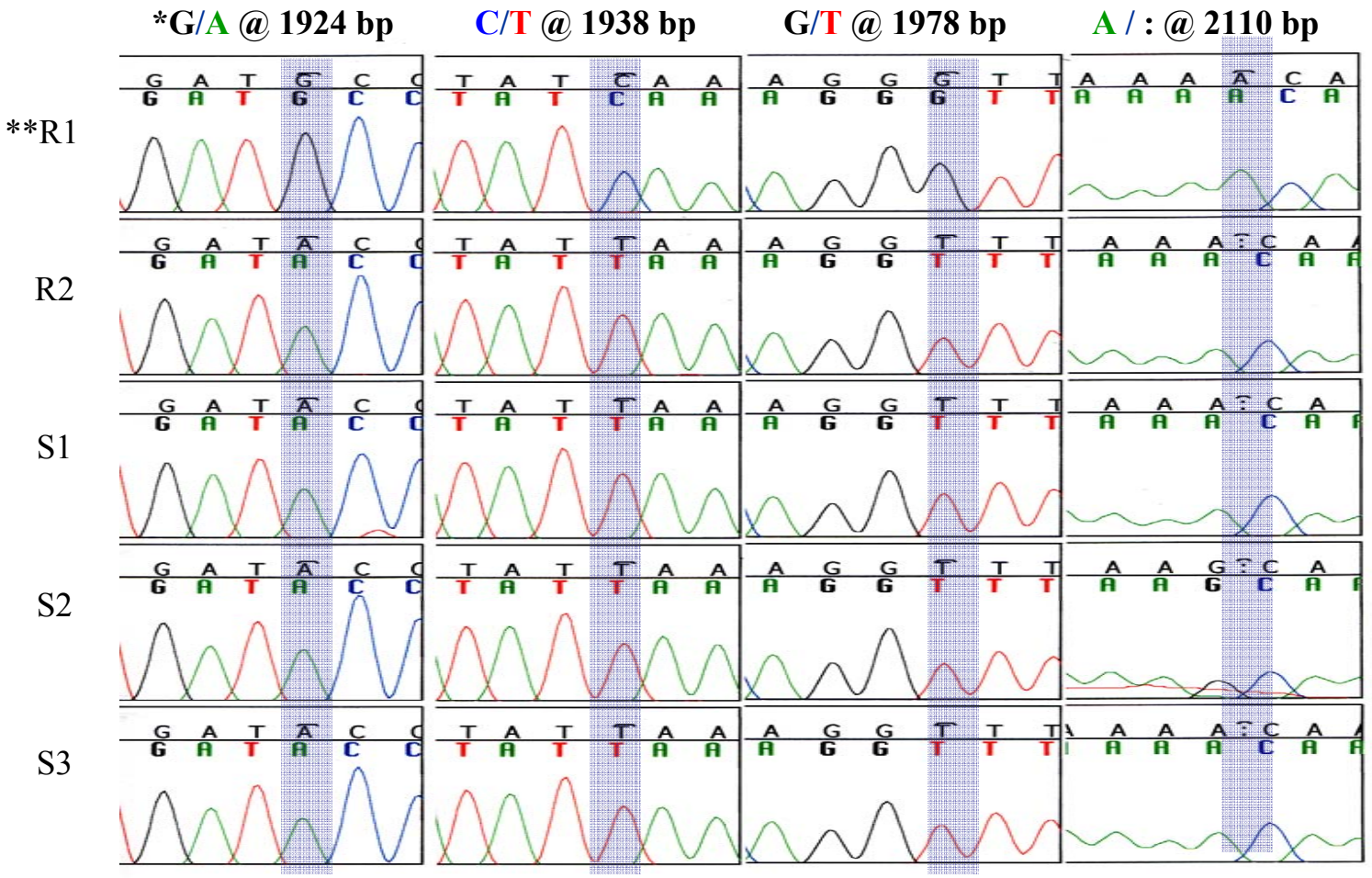

Fig. 8. Observation of the four consistent SNPs detected in clone E. *) Nucleotide replacement on resistance / susceptible @ the position in R2430E. **) Source of sequenced cloning insert.

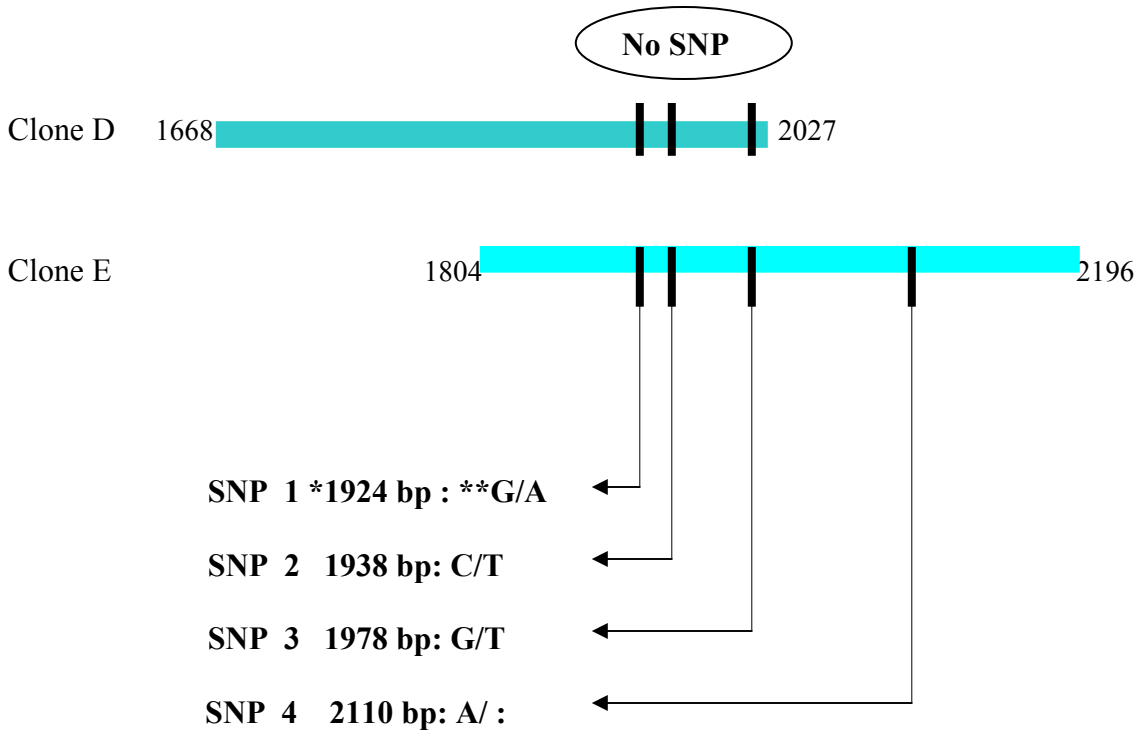

Fig. 9. Mismatches of SNPs between in clone D and E. Four SNPs, which found in clone E were not present in the same position of the sequence in clone D. *: Position of SNP found in clone E. **: nucleotide replace on resistant / susceptible.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The complete sequence of cDNA clone R2430E was 2217 bp and contained one putative ORF, which was from 36 bp to 749 bp. Nucleotide sequence homology showed very low similarity to nucleotide databases, but amino acid sequence of the putative ORF homology revealed high similarity to AAA ATPase containing von Willebrand factor type A (VWA) domain from *Magnetococcus* sp. MC-1 (106 bits). The function of AAA ATPase is nucleotide binding and it is now named AAA family, for 'A'TPases 'A'ssociated with diverse cellular 'A'ctivities. The key feature of this family is that they share a highly conserved region of about 220 amino acids that contains an ATP-binding site (Confalonieri and Duguet 1995).

Four probes, three representing different portions of R2430E and one for the entire R2430E, showed the same RFLP band pattern in both Florunner and NamaTAM peanut genotype on southern analysis. The only band specific for resistance (~8 kb) and a band for susceptibility (~7 kb) were observed (Fig. 4) and the observed RFLP pattern corresponded to the previous reports by Choi et al. (1999) and Church et al. (2000). As all probes showed the same RFLP pattern, it was concluded that the entire R2430E associates with RFLP bands linked the loci for resistance and susceptible. If different portions of R2430E hybridized to different RFLP bands, then it would have been

possible to design PCR primers that would amplify only the RFLP bands of interest, eg. the band specific to resistance.

Primer pairs derived from the sequence of R2430E were tested by PCR with peanut genomic DNA from Florunner and NemaTAM as a template to determine specific primer pairs for resistance. No primer pair amplified different size PCR products. This means that there was no primer pair specific for resistance. PCR amplification using 25 primer pairs and genomic DNA from Florunner and NemaTAM as a template amplified the same PCR products, which derived from cDNA clone R2430E as a template (No data shown). Therefore, it was assumed there is no intron in the genomic DNA region from Florunner and NemaTAM hybridizing with R2430E.

Because I was attempting to develop a SCAR for resistance based on a specific part of R2430E hybridizing to the resistance locus or with random primer pairs that hybridize to R2430E, it was necessary to sequence all RFLP bands to determine if any SNP, specific for the resistance phenotype, could be identified. Several non-consistent SNPs were found in each clone, which were derived from PCR amplification using primer pairs for different portions of R2430E and 7 subgenomic DNAs as a template (Table 6). It is believed that these non-consistent mismatches that were found in almost every clone were caused by sequencing errors. Additionally, four SNPs, which were observed consistently in all the sequence replicated clones of RFLP fragment R1 in clone E were not detected in clone D, which overlaps clone E.

There are two possibilities for this observation. First possibility is that mismatches between clone D and E were caused by PCR, cloning and sequence errors. One

additional independent PCR, cloning of the PCR product, and sequencing of the insert for clone E to determine a reason, which caused to sequence mismatches between clone D and E. In this last trial, no SNP in clone E was found. Thus, we concluded: (1) The sequence mismatches between clone D and E were caused by an error during PCR or cloning; (2) there is no polymorphism in the sequence region hybridizing with the probe. It may be outside of the region of the RFLP fragment hybridizing with R2430E. Therefore, additional sequencing of flanking region of the RFLP fragment hybridized to R2430E for resistance and susceptible genotype is required.

The approach for SNP detection in this study has potential errors associated with PCR, cloning and sequencing. To overcome these limits, recently a computer - based method has been developed to identify SNPs. Using a computer based method is possible to identify candidate SNP as well as small insertions/deletions from expressed sequence tag (EST) data (Batley et al. 2003). However, since peanut EST data is not available now, the best approach to reduce PCR, cloning and sequence errors was by increasing the number of replications of PCR, cloning, and sequence. In this study, PCR was carried out with three replications for each clone, but only one set of PCR products (7 templates × 5 primer pairs) (Table 5) was cloned. Then, sequencing was performed with three replications per clone. To determine whether a PCR, cloning and sequencing error caused the mismatches between clone D and E, sequencing of additional clones derived from another independent PCR is needed.

The other possibility is that there was no true SNP in the sequence of RFLP fragment hybridized to R2430E. According to the report by Paran et al. (1993), although SCAR

can be derived from RFLP probes, SCAR derived from RFLP probes may not be useful as PCR-based marker. The reason is that the polymorphism may be often outside the region hybridizing with the probe. In such case, the polymorphism will not be detected by primers obtained from the two ends sequences of RFLP probe. Our inability to detect SNP with the RFLP fragments may be due to the absence of a polymorphism in the region hybridizing to R2430E probe.

In addition to R2430E, other RFLP markers such as S1018E, R2545E can be used to develop SCARs. Based on a previous study, R2545E showed distinctive a band for resistant and susceptible alleles (Choi et al. 1999) and was used to screen individuals homozygous for resistance same as R2430E (Church et al. 2000). Also S1018E, which showed a very clear, distinctive band for resistance and susceptible alleles (Church 2002) can be used as a valuable marker and to convert to SCAR. Another PCR-based marker such as AFLP or SSR can be developed.

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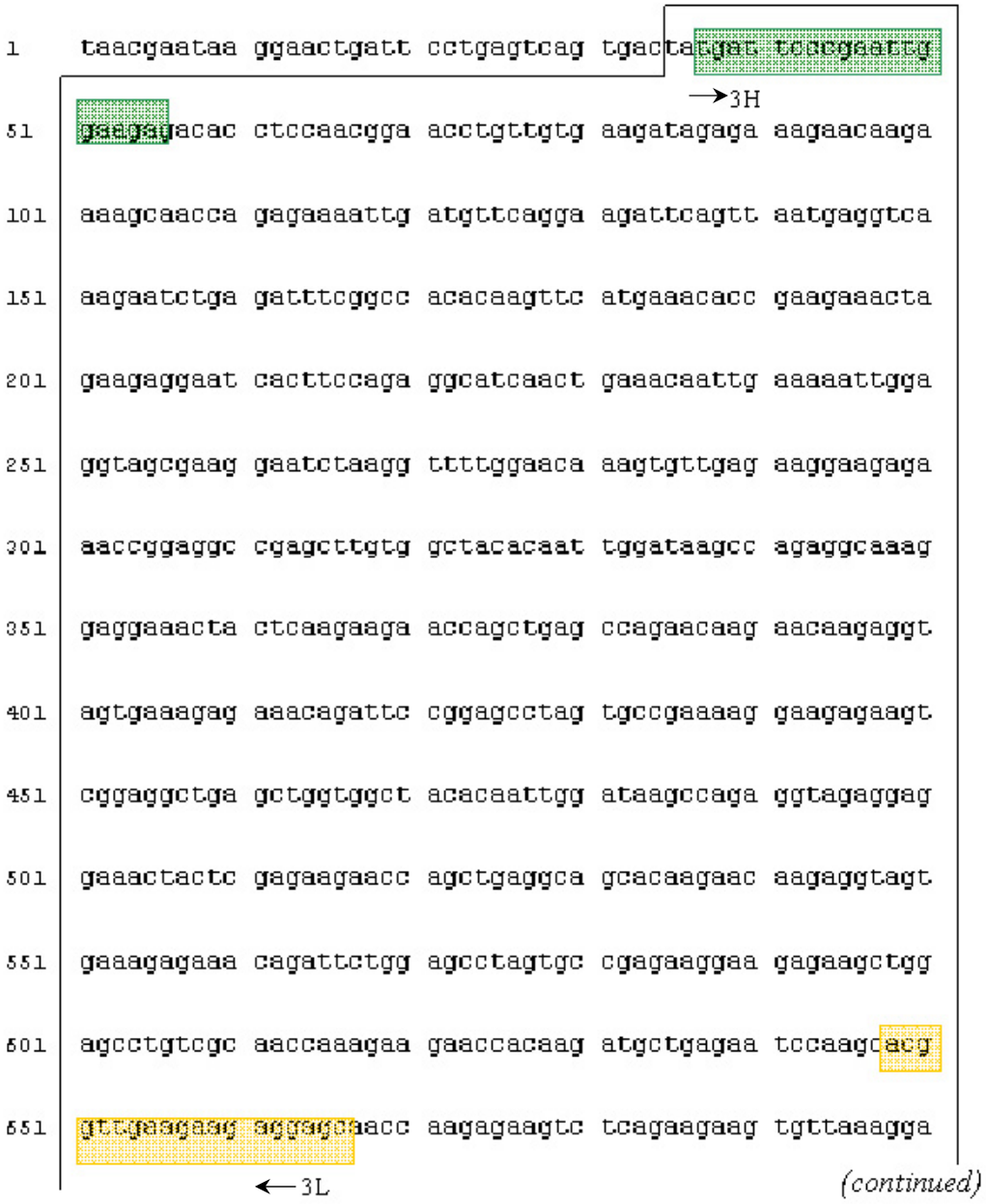
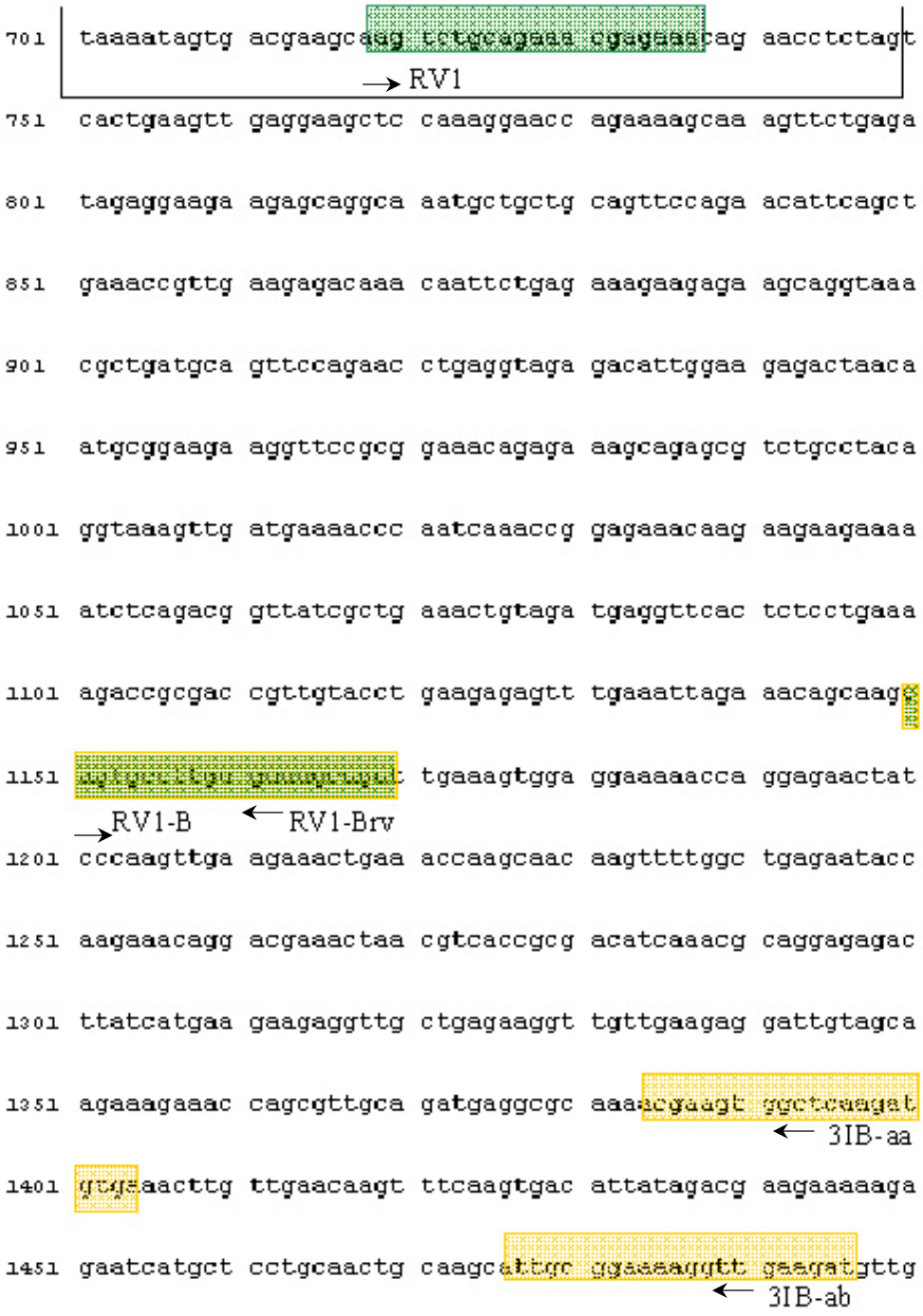
APPENDIX

Complete nucleotide sequence of R2430E





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851 gaaaccgttg aagagacaaa caattctgag aaagaagaga agcaggtaaa

901 cgctgatgca gttccagaac ctgaggtaga gacattggaa gagactaaca

951 atgcggaaga aggttccgcg gaaacagaga aagcagagcg tctgcctaca

1001 ggtaaagttg atgaaaaccc aatcaaaccg gagaaacaag aagaagaaaa

1051 atctcagacg gttatcgctg aaactgtaga tgaggttcac tctcctgaaa

1101 agaccgcgac cgttgtacct gaagagagtt tgaaattaga aacagcaagg

1151 [illegible] tgaaagtgga ggaaaaacca ggagaactat

1201 cccaagttga agaaactgaa accaagcaac aagttttggc tgagaatacc

1251 aagaaacagg acgaaactaa cgtcaccgcg acatcaaacg caggagagac

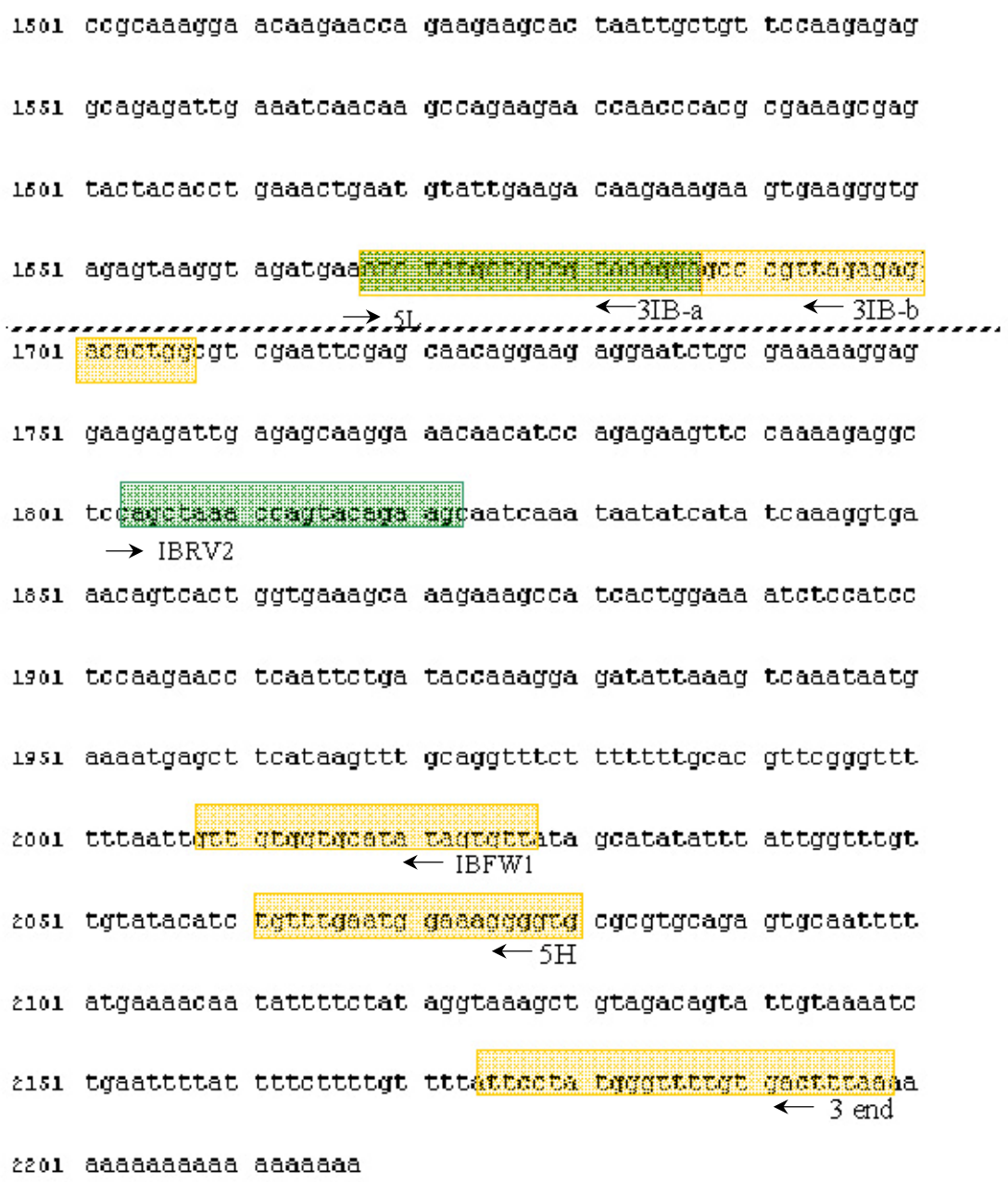
1301 ttatcatgaa gaagaggttg ctgagaaggt tgttgaagag gattgtagca

1351 agaaagaaac cagcgttgca gatgaggcgc aaaacgaagt ggctcaagat

1401 gtgaaacttg ttgaacaagt ttcaagtgac attatagacg aagaaaaaga

1451 gaatcatgct cctgcaactg caagcattgc ggaaaaggtt gaagatgttg

(continued)



The putative ORF is in the box. Sequencing primers are highlighted; greens indicate forward primers and yellows indicate reverse primers

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